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Inactivation of *Neosartorya fischeri* and *Paecilomyces variotii* on paperboard packaging material by hydrogen peroxide and heat

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ABSTRACT

This study reports on the influence of heat and hydrogen peroxide combination on the inactivation kinetics of two heat resistant molds: *Neosartorya fischeri* and *Paecilomyces variotii*. Spores of different ages (1 and 4 months) of these molds were prepared and *D-values* (the time required at certain temperature/hydrogen peroxide combination to inactivate 90% of the mold ascospores) were determined using thermal death tubes. *D-values* found for *P. variotii* ranged from 1.2 to 25.1 s after exposure to different combinations of heat (40 or 60 °C) and hydrogen peroxide (35 or 40% w/w) while for *N. fischeri* they varied from 2.7 to 14.3 s after exposure to the same hydrogen peroxide concentrations and higher temperatures (60 or 70 °C). The influence of temperature and hydrogen peroxide concentration on the *d*-values varied with the genus of mold and their ages. A synergistic effect of heat and hydrogen peroxide in reducing *D-values* of *Paecilomyces variotti* and *N. fischeri* has been observed. In addition to strict control of temperature, time and hydrogen concentration, hygienic storage and handling of laminated paperboard [illegible] All these measures [illegible]tic processing and [illegible]life.

1. Introduction

Aseptic processing consists in packaging a previously cold filled commercially sterile food into a sterilized package under aseptic conditions (Abreu and Faria, 2004; Kumar et al., 2008). Laminated paperboard, the most common material used for filling aseptic processed products, is stored as bobbins and the packages are formed after surface sterilization with the combination of hydrogen peroxide and heat followed by evaporation of the chemical by using dry heat (Von Bockelmann, 1982).

The contamination of paperboard material occurs, with few exceptions, due to the air and handling/contact with the packaging equipment (Värnamo, 1982). Although the level of contamination in paperboard material is of major importance for the success of aseptic processing, few studies report the microbial load found on these materials (Smith & Brown, 1980). Nevertheless, it is known that microbial loads such as 2–5 CFU cm^2/100 cm^2 can be found on paperboard material before its sterilization and transformation into packages (Värnamo, 1982; Von Bockelmann & Von Bockelmann, 1986).

Hydrogen peroxide (H_2O_2) is a strong oxidizing agent approved for packaging sterilization (FDA, 1984). Aseptic processing systems apply hydrogen peroxide in the range of 10–35% at room or higher temperatures, through spray or immersion methods (Smith & Brown, 1980). Although several factors may affect the effectiveness of the laminated paperboard sterilization process, it is known that heat plays a major role when applied along with hydrogen peroxide as it affects the formation of radicals that show sporicidal and bactericidal properties (Ansari & Datta, 2003; Juven & Pierson, 1996; Shin et al., 1994).

Hydrogen peroxide is known to be effective against a wide range of microorganisms (Hilgren, Swanson, Diez-Gonzalez, & Cords, 2009; Lin, Moon, Doyle, & McWatters, 2002; Podolak, Elliott, Taylor, Khurana, & Black, 2009; Venkitanarayanan, Zhao, & Doyle, 1999; Xu, Labuza, & Diez-Gonzalez, 2008). Some studies report on the inactivation of bacterial spores by hydrogen peroxide (Hilgren et al., 2009; Podolak et al., 2009; Xu et al., 2008), however, to the authors' best

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knowledge there is no research that regards the inactivation of heat resistant molds that may contaminate paperboard materials used in the aseptic processing. Heat-resistant molds such as *Byssochlamys*, *Neosartorya*, *Talaromyces*, *Eupenicillium* and anamorph with asexual spores such as *Paecilomyces* are well-known because of their high heat and chemical resistances (Salomão, Massaguer, & Aragão, 2008; Sant'Ana, Rosenthal, & Massaguer, 2009; Tournas, 1994; Tribst, Franchi, Cristianini, & Massaguer, 2009), involvement in food spoilage (Beuchat & Pitt, 2001; Tribst et al., 2009) and ability to produce mycotoxins (Beuchat & Pitt, 2001; Sant'Ana et al., 2010). These microorganisms are largely found in the soil (Beuchat & Pitt, 2001) and may be carried to food processing plants by dust and fruit surfaces (Beuchat & Pitt, 2001) resulting also in the contamination of packaging materials and foods.

In order to ensure the microbiological stability of aseptic processed products, the knowledge of inactivation kinetics of heat-resistant mold spores as affected by hydrogen peroxide concentration and heat is essential for the design of an effective package sterilization process. Therefore, this study aimed to determine the influence of combined heat and hydrogen peroxide on the inactivation kinetics of two heat resistant molds (*Neosartorya fischerii* and *Paecilomyces variotti*).

2. Material and methods

2.1. Microorganisms and preparation of spore suspensions

P. variotii F1A1 and a *N. fischeri* strains isolated from paperboard packages (Delgado, Sant'Ana, & Massaguer, 2000) and from spoiled tomato pulp (Baglioni, Gumerato, & Massaguer, 1999), respectively, were used in this study. These molds strains have shown either high heat (Baglioni et al., 1999) or hydrogen peroxide resistance (Delgado et al., 2000).

Mold spore suspensions were prepared as previously described (Delgado et al., 2000; Sant'Ana et al., 2009). It is known that age of spores influence molds' responses to activation or inactivation treatments as well their growth parameters (Blaszyk & Holley, 1998; Judet, Bensoussan, Perrier-Cornet, & Dantigny, 2008; Slongo & Aragão, 2006; Zimmermann, Miorelli, Massaguer, & Aragão, 2010). Therefore, to understand the influence of *P. variotii* and *N. fischeri* spores' age on heat and hydrogen peroxide resistance, two different spores suspensions were prepared by varying the incubation time: *I*: 1 month and *II*: 4 months. After preparation, suspensions were stored at 2–4 °C ± 1 °C and used within 6 months. All suspensions were standardized at 10^7–10^8 spores/mL and homogenized before using to avoid the presence of any clusters.

2.2. Preparation of hydrogen peroxide solutions

Hydrogen peroxide solutions were prepared by mixing food grade concentrated hydrogen peroxide (50% w/w) (Peróxidos do Brasil Ltda, Curitiba, Brazil) with sterile deionized water and used on the same day (Tsuchida & Tshuchido, 1997). The concentration of the diluted solutions was checked before their use as proposed by Buchen and Marth (1977). In addition, the concentration of the stock hydrogen peroxide was analyzed monthly by iodometric titration to ensure accuracy when preparing the solution for the inactivation experiments (Buchen & Marth, 1977). The pH of the 35 and 40% solutions were 1.75 and 1.54, respectively.

2.3. Inactivation of N. fischeri and P. variotii spores by hydrogen peroxide and heat combination

Inactivation kinetics of *N. fischeri* and *P. variotii* spores as affected by spores age (1 and 4 months), exposure to hydrogen peroxide (35 and 40% w/w) and heat (60 and 70 °C) was determined. Minimal hydrogen peroxide concentration (35%, w/w) and highest temperature value (70 °C) represent the common conditions used in aseptic packaging machines. On the other hand, the highest hydrogen peroxide concentration (40%, w/w) was selected to test an alternative temperature to compensate the decrease of one inactivating factor. As for *P. variotii* it was not possible to recover survivors after exposure at higher temperature (70 °C) and hydrogen peroxide concentrations (40%), an extra temperature condition (40 °C) was tested.

Sterile thermal death tubes (TDT; 8 mm external diameter, 6 mm internal diameter and 1 mm wall thickness) were filled with 1.8 mL of each hydrogen peroxide solution and 0.2 mL of *N. fischeri* or *P. variotii* spores (1 and 4 months). The final spore count after filling was 10^6–10^7 CFU/mL and the tubes were immediately sealed with the aid of a blowtorch (O_2/LGP). TDTs were disposed in a thermostatically controlled water bath (Polystat®, Poly Science, IL, USA, ± 0.1 °C) previously adjusted to the temperatures studied (40, 60 and 70 °C). Come-up times were determined as previously described by Sant'Ana et al. (2009) and considered in the inactivation experiments. After exposure to each temperature conditions, TDTs were cooled down in an ice bath and opened under aseptic conditions. Then, the hydrogen peroxide was neutralized by adding the content of the TDT tube to a sterile scrap tube (100 × 25 mm) and mixing with 19.5 mL of saline solution and 0.3 mL of catalase solution. The concentration of catalase solution (15.15 g/L) needed to neutralize hydrogen peroxide present in each TDT was previously determined (data not shown). Serial decimal dilutions were prepared and the counts made in duplicate MEA plates. Then, Petri dishes were incubated at 30 °C for 10 days and the counts were expressed as CFU/mL. Experiments were repeated twice and survival curves were drawn by the regression of the data of $\log_{10}$ population (survivors; CFU/g) against the time (s). *D-value* is defined as the time required at certain temperature/hydrogen peroxide to cause 1 $\log_{10}$ reduction in a microbial population.

2.4. Determination of hydrogen peroxide residuals after paperboard material disinfection in an aseptic processing plant

Although the disinfection process aims to inactivate molds spores to ensure paperboard package commercial sterility status, residuals of hydrogen peroxide above the maximum level established (0.5 ppm) should not be detected. An aseptic packaging unit (6000 packages/hour) located in a tomato pulp processing in the State of São Paulo, Brazil was used in this test. A concentration of 40% of hydrogen peroxide was tested to know whether residuals of hydrogen peroxide would be higher than the maximum level set by the FDA.

Twenty paperboard packages filled with water in a aseptic packaging machine were collected after paperboard disinfection with hydrogen peroxide solution (40%) at 71 C for 6 s. The packages were gently shacked for 15 s and opened using scissors. The water inside the package was collected and immediately analyzed for hydrogen peroxide using a reflectometer RQFlex Plus (Reflectoquant® - Merck, Darmstadt). Results were expressed as hydrogen peroxide residuals (mg/L).

2.5. Effects of hydrogen peroxide and heat on N. fischeri spores

A laser scanning confocal microscope model MCR 10024 UV visible (BioRad Laboratories, USA) equipped with Axovert Zeiss microscope and Lasersharp software was used to observe the changes in *N. fischeri* spores after exposure to heat/hydrogen peroxide treatments. This mold was chosen since it presents high heat/hydrogen peroxide resistance. One month age spores were

exposed to hydrogen peroxide solutions (35 and 40% w/w) at 70 °C/6 s. Spores were filtered in polycarbonate membranes (0.6 μm) using vacuum filtration after hydrogen peroxide neutralization with catalase solution (15.15 g/L). Spores were colored for 2 min with 2 mL of the fluorescent acridine orange colorant at 0.025% (w/v) prepared with 0.1 M citrate buffer (pH 6.5) (Pettipher, Mansell, McKunron, & Consuris, 1980). Spores were filtered again with 0.1 M citrate buffer (pH 6.5) to eliminate any colorant residuals. Then, the membranes were carefully taken with the aid of tweezers, placed inside Petri dishes and let drying at ambient conditions. Then, blades were placed onto the slides and observed in the confocal microscope with the aid of immersion oil. A magnification of 1000 times was used to focus the images and the final pictures were captured with zoom 3000–5000 times.

2.6. Experimental design and statistical analysis

The data on inactivation of *N. fischeri* and *P. variotii* spores by heat and hydrogen peroxide were treated using the response surface methodology (RSM). The quality of the fitted models was evaluated by ANOVA, based on the *F*-test and on the percentage of explained variance (R^2_{adj}), which provides a measurement of how much of the variability in the observed response values could be explained by the experimental factors and their interactions. Eq. (1) was used to fit the results:

$$Y = b_0 + b_1X_1 + b_2X_2 \quad (1)$$

Where: Y is the predicted response, b_0 is a constant, b_1, and b_2 are the regression coefficients and x_1, x_2 are the levels of the independent variables (hydrogen peroxide and temperature).

Experimental data were then fitted to the selected regression model to achieve a proper understanding of the correlation between each factor and different responses. This was obtained by estimating the numerical values of the model terms (regression coefficients), whose significance was statistically judged in accordance with the *t*-statistic at a confidence interval of 95%. ANOVA and *F*-test at a probability (P) of 0.05 were carried out to assess the global validity of the models to explain the actual relationship among factors and responses (Granato et al., 2010). To visualize the relationships between the responses and the independent variables, surface response plots of the fitted regression equations were generated using the statistical package Statistica 7.0 (Statsoft, USA).

3. Results and discussion

Although most of cases of spoilage of acidic fruit products are caused by heat resistant molds surviving to pasteurization treatments or underprocessing (Baglioni et al., 1999; Sant'Ana et al., 2009), the role of not properly sanitized aseptic package materials remains unknown. In a previous study (Delgado et al., 2000) we have reported on the occurrence of heat resistant molds such *P. variotii* and *T. flavus* on laminated paperboard material with counts between 0.71 and 1.02 CFU/cm^2. Although this material is sterilized by combining heat and hydrogen peroxide before contacting a food product, there is a chance for fails due to the occurrence of uncommon higher counts of molds or molds showing increased thermal or chemical resistance with potential to spoil foods. Despite this, to the best authors' knowledge, there is no report regarding the inactivation kinetics of heat resistant molds on laminated paperboard materials as influenced by a combination of heat and hydrogen peroxide.

D-values for *P. variotii* and *N. fischeri* as affected by heat and hydrogen peroxide concentration are presented in Table 1 and 2. It can be noticed that *N. fischeri* was more resistant to the treatments than *P. variotii*. After exposure at 60 °C and 35% of hydrogen peroxide, *D-values* for *N. fischeri* spores of 1 and 4 months were approximately 3 and 11 times higher than those for *P. variotii*, respectively. However, at 60 °C and 40% of hydrogen peroxide the *d*-values for *N. fischeri* spores were 1–2 times higher than *D-values* for *P. variotii*. This fact may indicate that the concentration of hydrogen peroxide approached a maximum value in which the increase in its concentration did not result in significant increase in the inactivation of microbial contaminants. Despite not improving the inactivation of molds, the increase of hydrogen peroxide concentration might result in corrosion of equipment and risks to workers.

Table 1
D-values for *P. variotii* (F1A1) at 1 and 4 months, as affected by hydrogen peroxide (%) and heat (°C).

Age (months)	H_2O_2(%)	Temperature (°C)	*D* value (s)
1	35	40	5.1
1	35	60	1.2
1	40	40	5.9
1	40	60	2.1
4	35	40	25.1
4	35	60	4.0
4	40	40	10.3
4	40	60	3.5

As can be seen in Table 1, *D-values* for *P. variotii* were determined only at 40 and 60 °C since at the most common temperature used for packaging sterilization (70 °C) it was not possible to recover survivors even after few seconds of exposure. However, we decided to use a low temperature to gather data on the inactivation of this microorganism as affected by heat and hydrogen peroxide as it has been continuously isolated from foods (Houbraken, Varga, Rico-Munoz, Johnson, & Samson, 2008; Piecková & Samson, 2000). Table 1 shows that for *P. variotti* of 1 month age, an increase of temperature from 40 °C to 60 °C resulted in a decrease of *D-value* of 3–4 times. However, an increase of hydrogen peroxide for a same temperature did not result the same effects. This is explained by the fact that for ascospores of *P. variotti* with 1 month age the regression equation was significant ($p = 0.01$) and the temperature was the main factor that decreased the *D-value*. This mathematical model was able to explain 93.1% of the variability in data (Table 3). For the same mold but with 4 months age, the increase of temperature led to decrease of *D-value* between 3 and 6 times. However, for a same temperature, the increase in hydrogen peroxide concentration only led to decrease in *D-values* when the experiment was carried out at 40 °C. At 60 °C the variation in the *D-value* was minimal. The mathematical model for 4 months aged ascopores of *P. variotti* was not able to explain the behavior of the system, once no significant terms were obtained by the regression analysis ($p = 0.20$).

For ascospores of *N. fischeri* with 1 month age (Table 2), an increase in temperature from 60 °C to 70 °C did not lead to significant reduction in the *D-value*, but the increase in the

Table 2
D-value for *N. fischeri* (control strain) at 1 and 4 months, as affected by hydrogen peroxide (%) and heat (°C).

Age (months)	H_2O_2 (%)	Temperature (°C)	*D* value (s)
1	35	60	13.9
1	35	70	11.4
1	40	60	4.9
1	40	70	3.6
4	35	60	14.3
4	35	70	11.7
4	40	60	3.9
4	40	70	2.7

Table 3
Regression analysis for the effect of hydrogen peroxide (%) and heat (°C) on inactivation of *P. variotii* and *N. fischeri* of 1 and 4 months ages.[a]

Microorganism	Age (months)	Regression equation	*p*-value (regression)	R^2 (%)	R^2_{adj} (%)
P. variotii	1	$y = 3.58 - 1.92\ T(°C)$	0.01	95.4	93.1
	4	$y = 10.70 - 3.82\ H_2O_2(\%) - 6.95\ T(°C)$	0.20	83.2	NA
N. fischeri	1	$y = 8.45 - 4.18\ H_2O_2(\%)$	0.01	94.4	91.6
	4	$y = 8.16 - 4.86\ H_2O_2(\%)$	0.03	95.7	93.6

[a] NA = not applicable since the model was not significant.

hydrogen peroxide concentration resulted in reduction of this parameter by three times. A similar behavior was observed for 4 months age *N. fischeri* ascospores and in both cases, the mathematical modeling of data indicated that the concentration of hydrogen peroxide seemed to be more effective and significant ($p = 0.03$ and $p = 0.04$, respectively). Regressions for 1 and 4 months ages ascospores of this mold were statistically significant ($p = 0.01$ and $p = 0.04$, respectively) with high adjusted determination coefficients ($R^2_{adj} = 91.6$) and ($R^2_{adj} = 93.6\%$), respectively) (Table 3). High values of R^2_{adj} are good indicators of suitability of models to describe the influence of the independent variables on the response (Granato et al., 2010), suggesting that the empirical models fit the actual data satisfactorily, defining the true behavior of the system well.

The results obtained in this study clearly show the synergistic effect of heat and hydrogen peroxide against ascospores of *P. variotti* and *N. fischeri*. *D-values* reported here (Tables 1 and 2) are by far lower than those found in our laboratory for these microorganisms. In preliminary experiments (not published), *D-values* for *N. fischeri* and *P. variotii* at 90 °C and 85 °C were 4.5 and 3.4 min when heated in distilled water, respectively. These values are in the range found in the literature for these heat resistant molds when only heat has been used as inactivating treatment. For instance, Salomão, Slongo, and Aragão (2007) reported D_{90} °*C* values for *N. fischeri* in papaya, pineapple and apple juices ranging from 1.5 to 2 min, while Kotzekidou (1997) reported D_{90} °*C* values ranging from 4.4 to 6.6 min. The survival of *P. variotti* with initial populations of 10^4–10^6 CFU/mL after heating at high temperatures such as 100 °C for different time intervals in water or foods has been reported by Piecková and Samson (2000).

In Fig. 1 it is shown the spores of *N. fischeri* of 1 month before (Fig. 1a) and after exposure to hydrogen peroxide at 35% (Fig. 1b) and 40% (Fig. 1c) at 70 °C/6 s. Spores exposed to 40% of hydrogen peroxide at 70 °C/6 s (Fig. 1c) were almost fully orange, indicating that their outer membranes were permeable to acridine orange and might have resulted in loss of viability in some extent. On the other hand, spores exposed to 35% of hydrogen peroxide at 70 °C/6 s (Fig. 1b) presented a light orange-yellow color that was much more intense in the extremities of spores, while most of control spores (Fig. 1a) presented green-colored outer and inner extremities. Although we only observed disruptions in outer membranes of spores after 2 h of exposure to hydrogen peroxide at 70 °C/6 s (data not shown), it should be noticed that inactivation of spores by hydrogen peroxide can take place without apparent morphological changes, such as lyse. This is explained by the fact that the hydrogen peroxide concentrations might not be enough to cause spore disruption, but can lead to oxidation of vital components for germination (Shin et al., 1994). It is known that hydrogen peroxide sporicidal and bactericidal properties result from its decomposition to water (H_2O) and nascent oxygen ([O]), with the late radical oxidizing microbial cells and spores components (Ansari & Datta, 2003; Juven & Pierson, 1996). *D-values* for spores of *N. fischeri* of 1 month were approximately 3 times lower after exposure to 40% solution of hydrogen peroxide than when it was 35%. Thus,

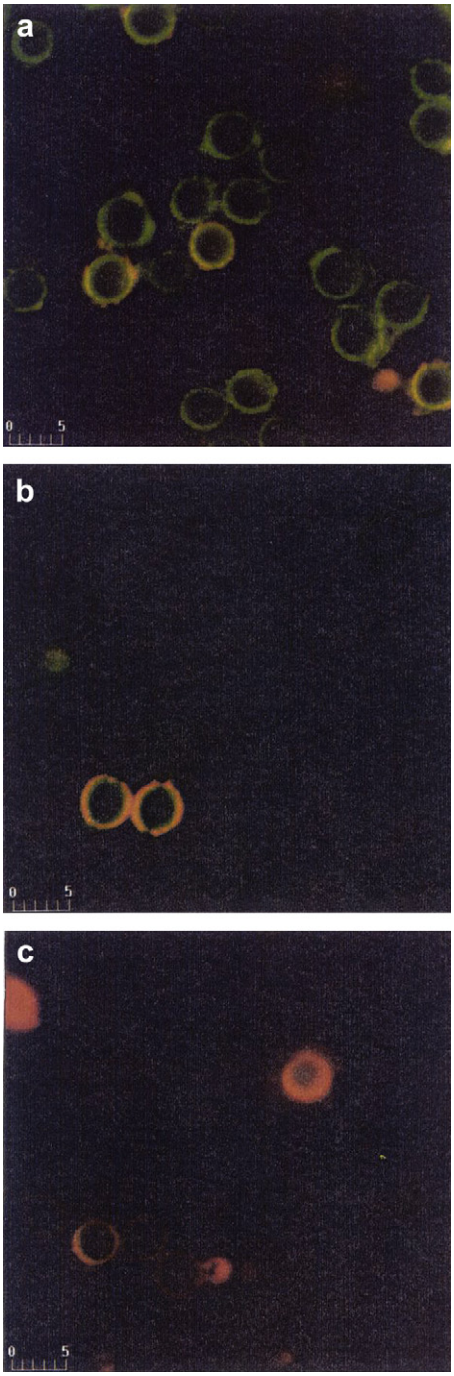

Fig. 1. Morphological modifications in spores of *N. fischeri* non-exposed to hydrogen peroxide (a), exposed at 35% at 70 °C/6 s (b) and 40% at 70 °C/6 s (c).

considering that *D* values for combined treatments are much lower than those for spores exposed to heat only, it can be concluded that penetration of hydrogen peroxide in the spore may be the main factor driving a higher inactivation for combined treatment. The sporicidal property of hydrogen peroxide is increased with the increase of temperature since the formation of hydrogen peroxide radicals is higher at temperatures ≥50 °C (Shin et al., 1994).

Tests in an aseptic processing plant have shown that residuals in the packages disinfected with 40% w/w of hydrogen peroxide at 70 °C and filled with water were in average 0.38 ± 0.19 ppm. Among the 20 packages analyzed, 14 and 12 samples presented levels of hydrogen peroxide ≤ 0.4 ppm and 0.3 ppm. However, six samples presented residual levels of hydrogen peroxide ≥ 0.5 ppm, which is the maximum residual level established by FDA (FDA, 1984). Taking into consideration only the microbiological results, one could indicate the use of a concentration of 40% w/w of hydrogen peroxide at 70 °C for 10–15 s in order to guarantee the inactivation of up to 10^3 spores/cm^2. However, residual levels of hydrogen peroxide in few packages subjected to this treatment might be higher than the maximum levels established by the FDA (0.5 ppm). Moreover, the safety of workers should be carefully considered since it is known that high levels of hydrogen peroxide can produce irritating vapors and residual levels can lead to degradation of nutrients such as vitamins that are susceptible to oxidation (Toledo, Escher, & Ayres, 1973) which brings the need to carefully consider the application of these results. Another alternative for achieving similar inactivation efficiency, but using a concentration of hydrogen peroxide of 35% w/w, would be the increase of hydrogen peroxide bath temperature (currently about 70 °C). However, in order to increase the hydrogen peroxide concentration, the residuals in packages and the safety of workers should be carefully considered since it is known that high levels of hydrogen peroxide can produce irritating vapors and residual levels can lead to degradation of nutrients such as vitamins that are susceptible to oxidation (Toledo et al., 1975.

The findings of this study show that a treatment combining heat (70 °C) and hydrogen peroxide (35%) for 6 s will guarantee packages' sterility if heat resistant molds are present in counts of up to 1 spore/100 cm^2. However, if counts than higher than 1 spore/100 cm^2 are present in package materials or if molds showing higher heat resistance than reported in this study are found, a treatment with 40% of hydrogen peroxide at 70 °C/10 s would be required to ensure a probability of survival of one in 10^4 packages. In addition to strict control of temperature, time and hydrogen concentration, hygienic storage and handling of laminated paperboard material must be considered to reduce the probability of package's contamination. All these measures together will ensure package's sterility that is imperative for the effectiveness of aseptic processing and consequently to ensure the microbiological stability of processed foods during shelf-life.

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